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Identification of 2,3-Dihydroxybenzoic Acid as a *Brucella abortus* Siderophore

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Brucella abortus **grown in low-iron medium or in the presence of iron chelators [ethylenediamine-di(*o*-hydroxyphenylacetic acid) and 2,2-dipyridyl] showed reduced cell yields and released a material positive in chemical and biological assays for catechols. This material was purified from culture fluids of *B. abortus* 2308 by chromatography on agarose-iminodiacetic acid-Fe^{3+} and identified as 2,3-dihydroxybenzoic acid (2,3-DHBA) by thin-layer chromatography, paper electrophoresis, and UV-visible nuclear magnetic resonance and mass spectroscopy. No other major catechols were observed at different stages of growth, and 2,3-DHBA was also produced upon iron limitation by representative strains of *B. abortus* biotypes 1, 5, 6, and 9. Both synthetic 2,3-DHBA and the natural catechol relieved the growth inhibition of *B. abortus* 2308 by ethylenediamine-di(*o*-hydroxyphenylacetic acid), and 2,3-DHBA promoted ^{55}Fe uptake by *B. abortus* 2308 by an energy-dependent mechanism. Two other monocatechols tested, 2,3-dihydroxybenzoyl-Ser and 2,3-dihydroxybenzoyl-Gly, also promoted ^{55}Fe uptake. More complex catechol siderophores (agrobactin and enterobactin), hydroxamate siderophores (aerobactin, ferrichrome, and deferriferrioxamine mesylate [Desferal]), and an EDTA-related siderophore (rhizobactin) failed to mediate ^{55}Fe uptake. *B. abortus* cells grown in low-iron medium or in medium with iron had similar rates of iron uptake when supplied with ^{55}Fe–2,3-DHBA, and the release of 2,3-DHBA under iron starvation was not associated with the expression of new outer membrane proteins. These results suggest an uptake system in which only the synthesis of the siderophore is regulated by the iron available for growth.**

Iron is the fourth most abundant element on earth. However, in most of the environments where microorganisms thrive, iron is either present as insoluble hydroxypolymers or tightly bound to organic molecules. Consequently, many microorganisms have developed systems to use low concentrations of free iron, to solubilize iron, or to remove it from environmental chelators (9). In the cells and body fluids of animals, free iron is kept at very low levels by iron-sequestering proteins (transferrin, lactoferrin, etc.), and therefore, it is postulated that iron acquisition systems are a prerequisite for bacterial pathogenicity (10, 25). Under iron-limiting conditions, many gram-negative bacteria release low-molecular-weight, high-affinity, iron-chelating agents (siderophores) and express specific receptor proteins to overcome the unavailability of iron (28, 29). Some bacteria can synthesize more than one type of siderophore, and many can utilize siderophores they themselves do not synthesize (28). In addition, iron acquisition by siderophore-independent mechanisms has been described for several species, including *Neisseria* spp. and *Haemophilus influenzae* (10).

The members of the genus *Brucella* are gram-negative, aerobic bacteria which multiply within macrophages. Although the information available on the role of iron in the biology of *Brucella* spp. is scanty, Waring et al. (47) and Evenson and Gerhardt (5) found that *Brucella suis* PS-1 and *Brucella abortus* 19 require iron for growth. In this paper we show that, under low-iron conditions, *B. abortus* excretes 2,3-dihydroxybenzoic acid (2,3-DHBA) and present evidence that it promotes iron uptake, thus acting as a siderophore.

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MATERIALS AND METHODS

Bacterial strains. The strains studied were *B. abortus* 2308 (smooth [S], virulent, biotype 1), *B. abortus* 19 (S, U.S. vaccine strain, biotype 1), *B. abortus* 544 CO_2 independent (S, virulent, reference strain of biotype 1), *B. abortus* B3196 (S, virulent, reference strain of biotype 5), *B. abortus* 8821 (S, virulent, biotype 6), *B. abortus* C68 (S, virulent, biotype 9), *B. abortus* RB51 (rough [R] mutant of strain 2308), and *B. abortus* 45/20 (R mutant of strain 45/0, biotype 1). Where indicated, *Escherichia coli* 0111 K-58 H2 (a pathogenic, enterobactin-producing strain [40, 41]) was employed as a control. All these strains were stored in skim milk at −70°C and, for routine use, maintained by serial transfer on tryptic soy agar (Oxoid Ltd., London, England).

Salmonella typhimurium LT2 *enb*-1 is a mutant with one of the last steps of the biosynthetic pathway of enterobactin impaired and whose growth is not stimulated by 2,3-DHBA (36); *S. typhimurium* LT2 *enb*-7 is a phenolate auxotroph that depends on 2,3-DHBA for enterobactin synthesis (36). Both mutants have been used previously in the bioassay of catechol siderophores (16, 32, 38, 48). *Aureobacterium flavescens* JG-9 ATCC 25091 is an auxotroph used in the bioassay of hydroxamate-type siderophores (16, 48, 49); this strain was maintained by serial transfer at room temperature on yeast extract-Casamino Acids-sucrose medium supplemented with 100 μg of deferriferrioxamine mesylate (Desferal; CIBA-GEIGY, S.A., Barcelona, Spain) per liter (52).

Growth media and conditions. (i) Low-iron medium. A modification of the *Brucella* synthetic liquid medium of Gerhardt et al. (7) was prepared as a quadruple-strength solution containing lactic acid (4.25 g), glycerol (37.8 g), monosodium glutamate (5.0 g), NaCl (7.5 g), K_2HPO_4 (10.0 g), $Na_2S_2O_3 \cdot 5H_2O$ (0.1 g), and yeast extract (0.5 g) in 250

ml of deionized, distilled water. Iron was extracted with 8-hydroxy-quinoline (oxine; E. Merck, Darmstadt, Germany) at a ratio of 25 mg/250 ml of quadruple-strength broth, and the metal-oxine chelates were removed with 20 ml of chloroform. After the oxine treatment was repeated two times, oxine was completely removed by repeated chloroform extraction (47). Residual chloroform was eliminated in a flash evaporator, the quadruple-strength broth was diluted four times and supplemented with $MgCl_2$ (10 mg/liter) and $MnCl_2$ (0.1 mg/liter), and the pH was adjusted to 6.8 with NaOH before autoclaving. The final iron content of the sterile low-iron medium was less than 0.9 μM as determined by atomic absorption spectrophotometry. Control medium was the oxine-extracted medium supplemented with $FeCl_3$ (Titrisol; E. Merck) to a final Fe^{3+} concentration of 15 to 60 μM.

(ii) Media with iron chelators. Tryptic soy broth (Oxoid) or the iron-supplemented controls of modified Gerhardt's medium were used. The iron chelators were 2,2-dipyridyl (DIP) (Sigma Chemical Co., St. Louis, Mo.) and ethylenediamine-di(*o*-hydroxyphenylacetic acid) (EDDA) (Fluka AG, Buchs, Switzerland). The latter was previously deferrated with HCl and acetone (40).

The bacteria were grown at 37°C with orbital shaking at 175 rpm, either in 2-liter Erlenmeyer flasks with 800 ml of broth or in 250-ml flasks with 75 ml of broth. Growth curves were obtained by monitoring the A_{540} of liquid cultures performed in sidearm flasks (Bellco Glass Inc., Vineland, N.J.). To obtain dry weights, cells were harvested by centrifugation, washed once with saline, resuspended in water, and freeze-dried.

Bioassays for siderophores. The presence of catechol-type siderophores was examined by a plate assay with the *S. typhimurium* auxotrophs as indicators in 250 μM EDDA-tryptic soy agar. The surface of the medium was inoculated with the indicator strain, and samples and controls were applied in paper disks (Concentration Disks; Difco Laboratories, Detroit, Mich.) impregnated with known amounts of the material to be tested. Plates were incubated at 37°C for 24 h. Bioassays for hydroxamate-type siderophores were performed by an analogous procedure with *A. flavescens* JG-9 as the indicator strain in sucrose-Casamino Acids-yeast extract agar with no Desferal (52). Incubations were performed at 26°C for 48 h with a solution of 1 μg of Desferal per ml as a control.

The ability of *B. abortus* to use siderophores was tested by an analogous procedure in modified Gerhardt's medium (see above) with EDDA (200 to 400 μM, final concentration) and 1% Noble agar (Difco) and using *B. abortus* 2308 as the indicator strain. Growth halos were measured after incubation at 37°C for 72 to 96 h.

Purification of the *B. abortus* siderophore. The spent low-iron liquid medium was concentrated 10-fold in a freeze-dryer, and insoluble materials were removed either by ultracentrifugation (86,000 × *g*, 12 h, 4°C) or by filtration in a Pellicon unit with a 100,000-molecular-weight-cutoff filter (PTHK 000 C5 cassette; Millipore Corp., Bedford, Mass.). A volume of 7 ml of the concentrate was applied to an agarose-iminodiacetic acid column (25 by 1.6 cm; Chelating Sepharose 6B; Pharmacia, Uppsala, Sweden) previously saturated with 5 mg of Fe^{3+} per ml of gel and equilibrated in 10 mM Tris-HCl, pH 7.5. The column was then washed with two volumes of 10 mM Tris-HCl, pH 7.5, and the material retained was eluted with 1 volume of 50 mM disodium EDTA. After the pH was adjusted to 2.0 with 6 N HCl, the retained and nonretained fractions were extracted thrice with ethyl acetate, and the organic phases were pooled and evaporated to dryness in a flash evaporator. The extract was resuspended in distilled water, ethanol, or ethyl acetate for subsequent analysis.

Cell envelope fractions. Bacteria were harvested by centrifugation and resuspended in a minimal volume consisting of 10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES; pH 7.5), 10 mM benzamidine (Sigma), 2 mM $MgCl_2$, and 100 μg of DNase and RNase per ml. Cells were disrupted by ballistic disintegration in an MSK cell homogenizer (B. Braun Mesulgen AG, Leinfelden, Germany), and the cell envelope fraction was sedimented by ultracentrifugation and characterized as described previously (27). Cell envelopes resuspended in 10 mM HEPES (pH 7.5)–0.05% sodium azide were stored at −20°C.

To obtain a fraction rich in outer membrane proteins, cell envelopes were extracted twice with 0.5% sodium *N*-lauroyl sarcosine (Sarkosyl; Sigma) in 10 mM Tris-HCl (pH 7.5) at a ratio of 5 mg of cell envelope protein per ml of extraction buffer. The Sarkosyl-resistant fraction, enriched in outer membrane proteins (27, 46), was washed once with 10 mM Tris-HCl (pH 7.5) and solubilized in 1% sodium dodecyl sulfate (SDS)–0.7 M 2-mercaptoethanol–10% glycerol–10 mM Tris-HCl (pH 6.8) for SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Analytical methods. (i) Chemical assays. Catechols and hydroxamates were determined colorimetrically by the Arnow (1) and Czaky (3) tests, respectively.

(ii) TLC. Thin-layer chromatography (TLC) was performed on 0.25-mm-thick silica gel 60 F_{254} or cellulose F plates (Merck) in one of the following solvent systems: benzene-acetic acid-water (125:72:3), butanol-pyridine-water (14:3:3), butanol-acetic acid-water (12:3:5), and chloroform-methanol (2:1). The plates were examined under UV light or sprayed with 0.12 N $FeCl_3$ in 0.1 N HCl to detect iron-binding compounds (31). To detect catechol-type compounds, they were sprayed with either the reagents of the Arnow assay (1) or 1% ferric ammonium citrate and then with 1% potassium ferricyanide (40). The standards were 2,3-DHBA, 2,4-DHBA, 2,5-DHBA, 2,6-DHBA, 3,4-DHBA, and 3,5-DHBA, all purchased from Fluka.

(iii) Paper electrophoresis. For high-voltage paper electrophoresis, a water-cooled flat-bed apparatus connected to a Savant 0-2 kV constant voltage supply (Savant Instruments Inc. Hicksville, N.Y.) was used. Samples were spotted onto Whatman no. 1 paper, and the mobilities of the compounds were determined by electrophoresis at 1,500 V in potassium phosphate buffer (0.1 M, pH 6.5). Spots were identified under UV light or by spraying with 0.12 N $FeCl_3$ in 0.1 N HCl. 2,3-DHBA was used as a standard.

(iv) ^{1}H NMR. Proton nuclear magnetic resonance (NMR) spectra were recorded on a Bruker 400 AM NMR spectrometer (Bruker Instruments Inc., Billerica, Mass.) at the University of California, Berkeley, NMR Facility. The samples were dissolved in deuterated methanol, and the chemical shifts were referred to the solvent peak at 3,300 ppm. 2,3-DHBA was used as a standard.

(v) EI-MS. Electron impact mass spectroscopy (EI-MS) was performed at the University of California, Berkeley, Mass Spectroscopy Facility with 2,3-DHBA as a standard.

(vi) SDS-PAGE. SDS-PAGE was performed in 12% acrylamide slabs with the discontinuous buffer system of Laemmli (20) and phosphorylase *b* (molecular weight, 92,000 [92K]), bovine serum albumin (66K), *E. coli* OmpF (38K), carbonic anhydrase (31K), and soybean trypsin inhibitor

(21K) as standards. Gels were stained for proteins with either Coomassie blue or alkaline silver (24).

(vii) Absorption spectra. Samples were resuspended in ethanol, and spectra were recorded with a UV-visible double-beam Zeiss DM4 spectrophotometer (C. Zeiss, Oberkochen, Germany) and a Servogor 319 recorder (BBC-Metrawatt/Goerz, Broomfield, Co.).

^{55}Fe uptake assay. The siderophores used in the ^{55}Fe uptake experiments were from J. B. Neilands' collection and are as follows: 2,3-dihydroxybenzoyl-serine (2,3-DHB-Ser) and 2,3-dihydroxybenzoyl-glycine (2,3-DHB-Gly) (monocatechols), enterobactin (cyclic catechol), agrobactin (linear catechol), aerobactin (citrate-hydroxamate), ferrichrome (trihydroxamate), and rhizobactin (EDTA-related siderophore) (for structures and detailed studies, see reference 50). Before the assay, stock solutions of ^{55}Fe complexes were prepared by adding $^{55}Fe^{3+}$ in 50 mM H_2SO_4 (specific activity, 19.63 Ci/g; Du Pont, NEN Research Products) to the ligand in ethanol and adjusting the pH to 7.4 with 1 M Tris-HCl. Stock solutions contained 20 μM $^{55}Fe^{3+}$ and 100 μM ligand.

When iron-starved cells were tested for iron uptake, they were grown in low-iron medium at 37°C with orbital shaking and harvested in either the logarithmic or the stationary phase of growth. When normal cells were used, they were grown in the same medium supplemented with iron. As a control, the supernatants were tested for a positive or negative catechol release by the Arnow assay (1). The cell pellets were washed thrice with ice-cold low-iron medium and resuspended in the same medium to an optical density at 600 nm of 0.2 to 0.3. A volume of 1.8 ml of the appropriate cell suspension was transferred to a 50-ml conical polypropylene tube and preincubated at 37°C for 45 min in a rotatory shaker at 350 rpm. The uptake was initiated by the addition of 200 μl of radiolabeled chelate (final concentration, 2 μM ^{55}Fe and 10 μM ligand). At regular intervals, aliquots (200 μl) were taken and filtered through 0.45-μm-pore-size Millipore HA filters previously soaked for 24 to 48 h in unlabeled ferric complex (to block nonspecific adsorption) and washed with 4 ml of ice-cold low-iron medium. Filter-bound cells were washed twice with 4 ml of ice-cold low-iron medium, placed in scintillation vials, and air dried overnight. A volume of 10 ml of scintillation fluid (CytoScint ES, ICN Biochemicals Inc.) was added, and the cell-associated radioactivity measured by liquid scintillation with a counting efficiency of 20 to 35% was determined for each experiment. Nonspecific adsorption of the ^{55}Fe complex to cells was corrected for by subtracting the radioactivity of the zero time sample from each value, and the results were expressed as picograms of ^{55}Fe per 10^8 cells. Energy inhibitors, where used, were added during the preincubation and uptake experiments.

RESULTS

Growth of *B. abortus* 2308 under iron-limiting conditions and catechol release. Table 1 summarizes the results of representative experiments in which *B. abortus* 2308 was grown under different conditions. The extraction with oxine (low-iron medium) had an adverse effect on growth that was iron related since it was reversed by this metal but not by magnesium or calcium. Growth was also reduced when EDDA or DIP was added to the low-iron medium supplemented with iron. This effect was manifested at concentrations of 50 μM (or more) either chelator.

The supernatants of *B. abortus* 2308 grown in the low-iron medium, supplemented or not with magnesium and calcium or grown in the presence of DIP and EDDA, showed a positive reaction in the Arnow assay for catechols (Table 1). On the other hand, a negative reaction was obtained in the Czaky assay for hydroxamates, even when the supernatants were concentrated 10-fold. When the supernatants of *B. abortus* 2308 grown in the low-iron medium were tested in bioassays, they reversed the EDDA inhibition and enhanced the growth of *S. typhimurium enb*-7 but not that of *S. typhimurium enb*-1. No growth stimulation of the hydroxamate-dependent auxotroph *A. flavescens* JG-9 was observed with the same preparations. The supernatants of the spent control medium supplemented with iron were negative in both the colorimetric assay and the bioassays for catechols.

TABLE 1. Growth and catechol production of *B. abortus* 2308 under iron-limiting conditions

Medium[a]	% Growth[b]	Catechol (nmol)/mg of cells[c]
Low iron	65	72
Low iron + Fe^{3+}	100	0
Low iron + Fe^{3+} + 200 μM EDDA	42	106
Low iron + Fe^{3+} + 300 μM DIP	23	168
Low iron + Mg^{2+}	67	54
Low iron + Ca^{2+}	69	79

[a] The indicated cations were added as the chloride forms to a final concentration of 5 μg/ml.
[b] Expressed as the percentage of cell dry weight with respect to the low-iron medium supplemented with Fe^{3+}.
[c] Determined by the Arnow test with 2,3-DHBA as a standard.

Characterization of the catechol produced by *B. abortus*. The Arnow-positive material present in the supernatants of *B. abortus* 2308 was concentrated by a triple ethyl acetate extraction. Analysis by TLC of this crude extract showed a major component reacting with Fe^{3+} (Fig. 1, lane C) and the

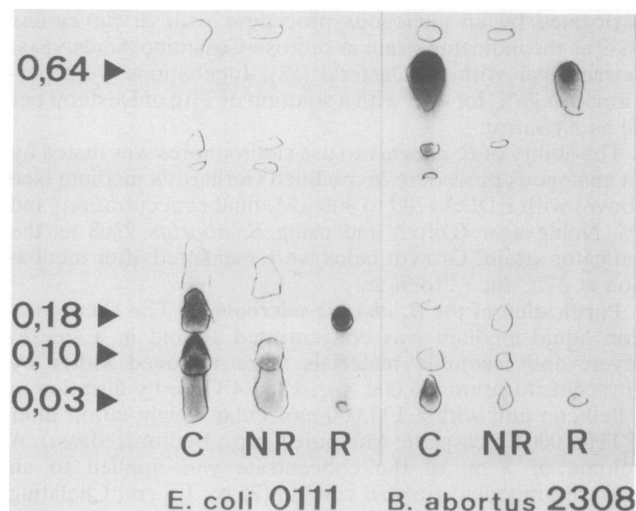

FIG. 1. Analysis by TLC on silica gel 60 F_{254} of the culture fluids of cells grown in low-iron medium (C) and the corresponding nonretained (NR) and retained (R) fraction obtained by agarose-iminodiacetic acid-Fe^{3+} chromatography. The plates were developed in benzene-acetic acid-water, the edge of the spots visible under UV light were marked, and the plates were sprayed with $FeCl_3$ in HCl. R_f values are indicated on the left.

reagents of the Arnow assay. The R_f of this major component (0.64 in benzene-acetic acid-water) was different from those of the catechols of stationary-phase *E. coli* (0.03, 0.10, and 0.18 in the same solvent system) (Fig. 1, lane C) but identical to that of synthetic 2,3-DHBA in the two types of gels and four solvent systems used (Table 2). In addition to this major component, some but not all *B. abortus* 2308 preparations showed a minor spot of R_f 0.02 in benzene-acetic acid-water which reacted with $FeCl_3$ and the reagents of the Arnow assay.

The components with iron affinity present in the supernatants of *B. abortus* 2308 grown in low-iron medium were purified by affinity chromatography on agarose-iminodiacetic acid-Fe^{3+}. TLC analysis of the fraction retained by the column showed only the catechol of R_f 0.64 (Fig. 1, lane R). The retained fraction stimulated the growth of *S. typhimurium enb*-7 but not of strain *enb*-1. Synthetic 2,3-DHBA was also retained by the agarose-iminodiacetic acid-Fe^{3+}, and it could not be resolved from the *B. abortus* 2308 major catechol when they were mixed and analyzed by TLC. Moreover, synthetic 2,3-DHBA and the retained fraction of *B. abortus* 2308 supernatant had the same mobility in paper electrophoresis and the same UV-visible absorption spectra (absorption maximum at 320 nm). On the other hand, the catechol obtained by affinity chromatography on agarose-iminodiacetic acid-Fe^{3+} was different from other DHBA isomers on the basis of their R_f, UV-visible absorption spectra, and reactivity with catechol-specific reagents (not shown).

Table 3 summarizes the results of the ^1H NMR analysis of the component retained by the agarose-iminodiacetic acid-Fe^{3+} column. The chemical shifts and coupling constants were identical to those of synthetic 2,3-DHBA and in accord with published values (6, 21, 34). Finally, EI-MS revealed a molecular ion $[M]^+$ at m/z 154. Other peaks were: m/z (relative intensity) 136(79) and 108(28), corresponding to $[M-H_2O]^+$ and $[M-CH_2O_2]^+$, respectively. Therefore, by this method of analysis the purified catechol was also indistinguishable from synthetic 2,3-DHBA.

Catechol production by *B. abortus* 2308 at different stages of growth. The production of catechols during the growth of *B. abortus* 2308 was examined by the colorimetric assay of Arnow and by TLC of ethyl acetate extracts obtained directly from the spent low-iron medium. Catechol release started at the beginning of growth (Fig. 2), reached a maximal concentration at the end of the logarithmic phase (255 nmol/ml after 66 h of incubation), and declined during the stationary phase (130 nmol/ml after 96 h). TLC analysis of the ethyl acetate extracts of the culture fluids taken at different intervals did not show iron-reacting compounds different from the catechol of R_f 0.64 observed in the experiments described above. When incubation was extended for longer periods, a dark brown material built up in the culture fluid. This material could not be dialyzed out by using standard dialysis membranes (cutoff, 10,000) and no attempts were made to characterize it. TLC analysis of the ethyl acetate extracts of the culture fluids taken at different intervals did not show iron-reacting compounds different from those observed in the experiments described above.

TABLE 2. R_f values of $FeCl_3$-reacting components released by *B. abortus* 2308 grown in low-iron medium

Gel + solvent system	R_f value[a]		
	Minor	Major	2,3-DHBA[b]
Cellulose + benzene-acetic acid-water	ND[c]	0.61	0.61
Silica + benzene-acetic acid-water	0.02	0.64	0.64
Silica + butanol-pyridine-water	0.00	0.53	0.53
Silica + butanol-acetic acid-water	0.23	0.85	0.85
Silica + chloroform-methanol	0.07	0.33	0.33

[a] Minor and major refer to the relative amounts of $FeCl_3$-reacting components as revealed by the stain (Fig. 1).
[b] Synthetic 2,3-DHBA.
[c] ND, not detected.

TABLE 3. Proton NMR chemical shifts and coupling constants of the *B. abortus* 2308 catechol purified by affinity chromatography on agarose-iminodiacetic acid-Fe^{3+}

Resonance	Chemical shifts (ppm)	Coupling constants (Hz)	Multiplicity[a]	Integral
ortho-H	7.34	*Jop* = 1.5, *Jom* = 8.0	q	1.0
meta-H	6.71	*Jmo* = 7.9, *Jmp* = 7.9	t	1.0
para-H	6.98	*Jpo* = 1.5, *Jpm* = 7.9	q	1.0

[a] t, triplet; q, quartet.

Siderophore activity of the *B. abortus* catechol. Bioassays in EDDA–low-iron medium with *B. abortus* 2308 as the indicator strain were performed with the catechol purified by the agarose-iminodiacetic acid-Fe^{3+} method. Enterobactin, commercial 2,3-DHBA, and Desferal were used as controls. The results showed that *B. abortus* 2308 was stimulated by the major catechol and by synthetic 2,3-DHBA but not by Desferal or enterobactin. Moreover, when adjusted to the same concentration by the colorimetric assay of Arnow and supplied with the same amount of iron (0.25 μg of Fe per nmol of catechol), the halos of the *B. abortus* catechol and of 2,3-DHBA were of the same size. On the other hand, controls with paper disks impregnated with the same amount of iron either did not stimulate growth or were surrounded by narrow growth halos, thus demonstrating that under the conditions of the assay the bacteria were using the 2,3-DHBA-iron chelate and not free iron.

2,3-DHBA-promoted ^{55}Fe uptake. Logarithmic-phase cells of *B. abortus* demonstrated iron uptake when it was supplied

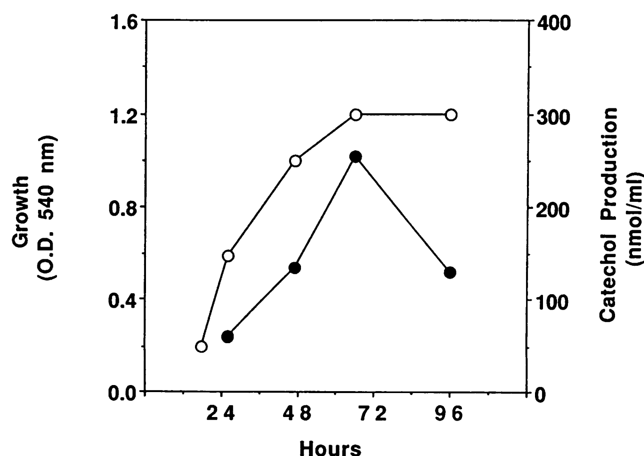

FIG. 2. Production of catechols by *B. abortus* 2308 during growth. Cells were grown in low-iron medium in sidearm flasks for the determination of the optical density (○), and aliquots were removed at the indicated times for the determination of the catechol content by the Arnow test (●).

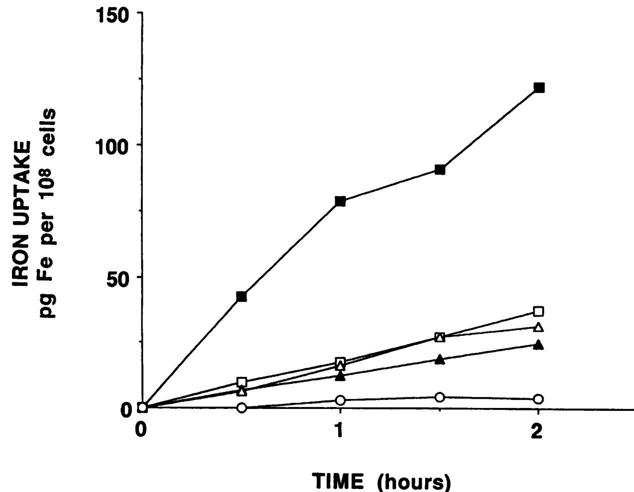

FIG. 3. 2,3-DHBA-promoted iron uptake. *B. abortus* 2308 logarithmic-phase cells were preincubated and assayed at 37°C (■), at 0°C (○), or at 37°C in the presence of 5 mM potassium cyanide (△), 5 mM sodium arsenite (▲), or 5 mM dinitrophenol (□). The uptake mixture contained 2 μM $^{55}Fe^{3+}$, 10 μM 2,3-DHBA, and cells at an optical density at 600 nm of 0.2 to 0.3. The data are representative of three separate experiments.

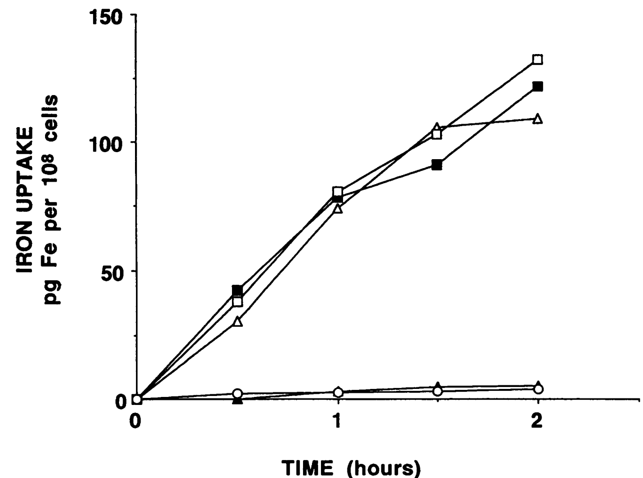

FIG. 4. Catechol-siderophore-mediated iron uptake in *B. abortus* 2308. The siderophores tested were 2,3-DHB-Ser (△), 2,3-DHB-Gly (□), enterobactin (○), agrobactin (▲), and 2,3-DHBA (■). The cells used were logarithmic-phase cells preincubated and assayed at 37°C. The uptake mixture contained 2 μM $^{55}Fe^{3+}$, 10 μM siderophore, and cells at an optical density at 600 nm of 0.2 to 0.3. The data are representative of three separate experiments.

in the form of ^{55}Fe-DHBA (Fig. 3). An almost complete blockage of transport was obtained when the cells were kept at 0°C during the experiment (Fig. 3). In addition, the uptake was 50 to 60% inhibited by 0.1 mM potassium cyanide or 0.1 mM sodium arsenite and about 28% by 0.1 mM dinitrophenol. The inhibition was 70 to 80% when the concentration of the energy inhibitors was 5 mM (Fig. 3). Experiments performed with stationary-phase cells showed a 37% reduction in the rate of iron uptake with respect to that obtained with logarithmic-phase cells.

To examine whether the 2,3-DHBA-iron uptake system was inducible, the experiments were repeated with logarithmic-phase cells grown in low-iron medium supplemented with iron. The results showed that the cells grown under such conditions took up amounts of iron (130.7 pg of Fe per 10^8 cells in 2 h) similar to those found with the cells grown in the absence of iron.

The specificity of the uptake with regard to the siderophore was examined. Two monocatechols tested, 2,3-DHB-Ser and 2,3-DHB-Gly, promoted ^{55}Fe uptake as efficiently as 2,3-DHBA (Fig. 4). On the contrary, two complex catechol siderophores, enterobactin and agrobactin (Fig. 4); the hydroxamate siderophores (aerobactin, ferrichrome and Desferal); and the EDTA-related siderophore rhizobactin did not promote iron uptake. No uptake was observed when *B. abortus* 2308 was incubated in the absence of siderophores.

Production of catechols by representative strains of *B. abortus*. Catechol production in the low-iron medium by *B. abortus* strains 544 (biotype 1), B3196 (biotype 5), 8821 (biotype 6), and C68 (biotype 9), as well as by strain 19 (vaccine strain of biotype 1) and the rough strains 45/20 and RB51, was examined. Strains 544, B3196, 8821, C68, 19, and RB51 released catechols that could be detected colorimetrically, with values ranging from 270 to 36 nmol of catechol per ml of broth after 72 h of incubation. These results were confirmed by bioassays with *S. typhimurium enb*-7, and TLC analysis demonstrated only the presence of the catechol of R_f 0.64 (benzene-acetic acid-water) found in the supernatants of *B. abortus* 2308. *B. abortus* 45/20 did not produce levels of catechol detectable by the colorimetric method, but bioassays revealed small amounts of catechol in the corresponding supernatant fluids.

Outer membrane profile of cells grown under iron-limiting conditions. Figure 5 presents an SDS-PAGE analysis of the extracts enriched in outer membrane proteins of *B. abortus* RB51 and *E. coli* 0111. No differences were observed in the protein profiles of *B. abortus* RB51 grown under normal or iron-limiting conditions, and this result was confirmed with strains 2308 and 45/20. No iron-repressed outer membrane proteins were observed in tryptic soy broth supplemented

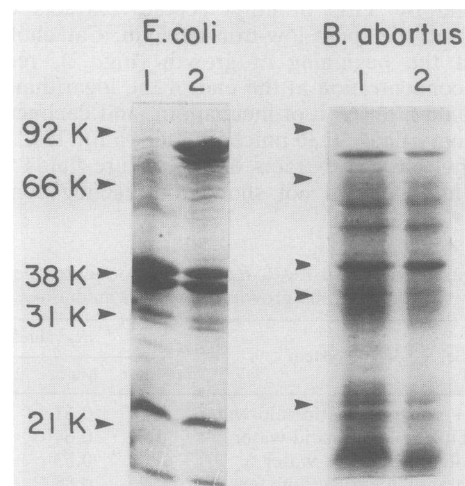

FIG. 5. SDS-PAGE analysis of the Sarkosyl-resistant fraction of the cell envelopes of *E. coli* 0111 and *B. abortus* RB51 grown in low-iron medium supplemented with iron (lanes 1) and low-iron medium (lanes 2). Arrowheads mark the positions of the molecular weight standards.

with either DIP or EDDA or when *B. abortus* RB51 and 2308 were serially subcultured seven times in low-iron medium to deplete possible intracellular iron pools. On the other hand, a new set of high-molecular-weight proteins was observed in the outer membrane of *E. coli* 0111 grown under the same conditions (Fig. 5). These analyses also showed that *Brucella* outer membrane proteins of group 1 (apparent molecular weight, 86,000 to 90,000 [46]) had an electrophoretic mobility similar to that of the iron-repressed *E. coli* outer membrane proteins.

DISCUSSION

The results of the present work show that *B. abortus* is able to synthesize and release catechols under the influence of the iron available for growth. The *B. abortus* catechol was purified and characterized by TLC, paper electrophoresis, UV-visible spectroscopy, ^{1}H NMR, and EI-MS, and this set of analytical data demonstrates that it is 2,3-DHBA. In addition, it was found that the *B. abortus* catechol stimulated the growth of *S. typhimurium enb*-7 but not the growth of the *enb*-1 strain. Since *enb*-7 is a phenolate auxotroph and *enb*-1 is unpaired in a later step of the enterobactin pathway (36), the results of the biological tests are fully consistent with those of the chemical characterization.

In *B. abortus* 2308, a minor Arnow-positive compound of very low R_f was observed occasionally in some batches of spent low-iron medium. During the growth of *E. coli* under iron-limiting conditions, 2,3-DHBA is released early to be gradually substituted in the medium by enterobactin and the linear esters of 2,3-DHB-Ser (31). However, a similar situation in *B. abortus* is unlikely since an increase during growth of the minor Arnow-positive compound of *B. abortus* 2308 was not observed and *B. abortus* was unable to use iron when supplied as the enterobactin chelate. It is also significant that production of catechols different from 2,3-DHBA was not detected in any of the six strains in which TLC analysis was performed. Since quinones and brown polymers are produced from catechols by air oxidation at neutral or alkaline pH (30), the minor Arnow-positive compound could be an artifact brought about by the high oxygen tension and neutral pH necessary to grow large amounts of *B. abortus*. This could also be the origin of the brown compound that built up in the low-iron medium of *B. abortus* after protracted incubation and one of the reasons for the declining of 2,3-DHBA during the stationary phase.

The observation that 2,3-DHBA relieved the growth inhibition caused by EDDA, a type of activity generally regarded as indicative of a true siderophore (30), strongly suggests that 2,3-DHBA is able to deliver iron to *B. abortus* cells and, therefore, that it is not simply a secondary metabolite. This interpretation is consistent with the results of the ^{55}Fe–2,3-DHBA experiments that demonstrated an uptake dependent on metabolic energy and influenced by the physiological state of the cells. Even though the rate of iron uptake exhibited by *B. abortus* was slower than those found in other bacteria (12–14, 18, 37), this could be due to the slow metabolism characteristic of *B. abortus*. In our experiments, the doubling time of *B. abortus* was 2.5 to 3.5 h, a value that is in perfect agreement with published data (39). Also, the likely absence of an outer membrane receptor (see below) could make the uptake relatively inefficient.

Production of 2,3-DHBA under iron-limiting conditions has been reported for several bacteria (2, 6, 17, 19, 31, 44), and it has been reported that simple monocatechols can promote iron uptake. In *E. coli*, 2,3-DHB-Ser acts as a siderophore (13) and the 2,3-DHBA iron chelates promote growth and penetrate through the outer membrane via Cir and Fiu, even though iron is then transferred to enterochelin or to the lineal esters of 2,3-DHBA-Ser (12, 13). An uptake of iron mediated by 2,3-DHBA and 2,3-DHB-Gly has also been shown for *Bacillus subtilis* (35). Likewise, 2,3-DHBA and 2,3-DHB-Lys are used efficiently as siderophores by *Erwinia chrysanthemi* (33). Similar findings with respect to 2,3-DHBA have been described in *Acinetobacter calcoaceticus* (44). The relative lack of specificity of the *B. abortus* system, that was able to use other simple monocatechols, is not surprising, since it has been observed in other bacteria (13, 33, 35). The system, however, was not able to take up iron bound to the complex catechols enterobactin and agrobactin or to noncatechol siderophores. Some agrobacteria are phylogenetically related to *Brucella* spp. (26) and produce catechol-type siderophores, but it seems that the respective iron acquisition systems have evolved differently, perhaps as an adaptation to the hosts with which they are associated (26). In this regard, it is interesting that *B. abortus* can produce 2,3-DHBA because this catechol has biological activities, such as inhibition of membrane peroxidation (8), that could be relevant for an intracellular parasite. Feistner and Beaman (6) have suggested that the production of 2,3-DHBA by *Nocardia asteroides* could play a role in protecting this bacterium against the oxygen-dependent killing mechanism of phagocytes, and the same possibility exists in *B. abortus*. Even though strains 2308 and 544 (virulent), strain 19 (attenuated), and strains RB51 and 45/20 (avirulent) all produced 2,3-DHBA, strains RB51 and 45/20 are rough mutants, and it is well known that, in *B. abortus*, smoothness is required for full pathogenicity. Site mutagenesis carried out on virulent strains would help to investigate the role of 2,3-DHBA in *B. abortus* pathogenicity.

No changes were detected in the outer membrane protein profile of *B. abortus* grown under iron-limiting conditions and, although *B. abortus* group 1 proteins (46) had an electrophoretic mobility similar to that of the iron-repressed *E. coli* outer membrane proteins, group 1 has been consistently observed under normal conditions (51). Moreover, our results are in agreement with that of Marquis (22), who found no changes in the outer membrane proteins of *B. abortus* 2308 grown with 200 μM DIP. The possibility that the iron levels were not low enough to trigger the expression of new outer membrane proteins seems unlikely. First, in our experiments growth was clearly reduced, showing that the conditions were those under which the iron acquisition systems should show its adaptational value. That it was so is illustrated by the fact that synthesis of catechol by *B. abortus* occurred in the low-iron media but not in the media with available iron. Second, the results did not change after serial subculture of *B. abortus* in low-iron media. Third, in the *E. coli* controls, the conditions used with *B. abortus* caused the simultaneous synthesis of the siderophore and the outer membrane proteins that act as receptors (10). Fourth, the lack of an inducible receptor is also suggested by the ^{55}Fe uptake experiments because the cells grown in low-iron medium and in the same medium plus iron showed similar rates of iron uptake.

Since many gram-negative bacteria present outer membrane proteins that are iron repressed (15, 29, 42, 43, 45, 48, 49), their absence in *B. abortus* is intriguing. It is possible that the receptor is not iron regulated, and in this case group 1 proteins would be a candidate, but it would be difficult to understand why expression of the siderophore is under iron repression while that of the receptor is not. Alternatively, a

system without an outer membrane receptor could be operative, since gram-negative bacteria take up many substrates by receptor-independent pathways. A porin pathway cannot be discarded because, in contrast with the chelates formed by complex catechols, the bidentate ligands of simple catechols are small enough to penetrate through the porins of *B. abortus*, whose exclusion limit is similar to that of *E. coli* OmpF (4). In addition to porins, two receptor-independent pathways have been described: the self-promoted pathway for polycations and the hydrophobic pathway (11). Because of the shielding of the outer membrane by divalent cations, the hydrophobic pathway is seldom operative in wild-type strains of gram-negative bacteria (11). However, it has been shown that the envelopes and lipopolysaccharide of *B. abortus* are not stabilized by divalent cations (27), and we have found recently that hydrophobic fluorescent probes readily penetrate the outer membrane of smooth *Brucella* spp. (23). Thus, the hypothesis that the moderately hydrophilic 2,3-DHBA chelates could penetrate through the outer membrane of *B. abortus* by a hydrophobic pathway cannot be ruled out at present.

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